Förster resonance energy transfer (FRET) is a mechanism where energy is transferred from an excited donor fluorophore to adjacent chromophores via non-radiative dipole-dipole interactions. FRET theory primarily considers the interactions of a single donor-acceptor pair. Unfortunately, it is rarely known if only a single acceptor is present in a molecular complex. Thus, the use of FRET as a tool for measuring protein-protein interactions inside living cells requires an understanding of how FRET changes with multiple acceptors. When multiple FRET acceptors are present it is assumed that a quantum of energy is either released from the donor, or transferred to only one of the acceptors present. The rate of energy transfer between the donor and each specific acceptor (k_{DA}) can be measured in the absence of other acceptors, and these individual transfer rates can be used to predict the ensemble FRET efficiency. The generality of this approach was tested by measuring the ensemble FRET efficiency in two constructs, each containing a single fluorescent-protein donor (Cerulean) and either two or three acceptors (Venus). FRET transfer rates between individual donor-acceptor pairs were measured by systematically introducing point mutations to eliminate the chromophores of the other acceptors. We find that the amount of FRET with multiple acceptors is significantly greater than predicted by the sum of the individual transfer rates. We conclude that either an additional energy transfer pathway exists when multiple acceptors are present, or that a theoretical assumption that the prediction calculation is based on is incorrect. These possibilities will be discussed.

3026-Pos

Fluorescence Fluctuation Spectroscopy in the Presence of Hydrodynamic Flow to Determine Protein Stoichiometry at Ultra Low Concentrations

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Fluorescence fluctuation spectroscopy (FFS) provides information about transport parameters, concentration, and interactions of fluorescently labeled molecules. One important FFS parameter is brightness which provides information about the stoichiometry of protein complexes. Here we consider the application of FFS to large protein complexes, such as viruses. FFS measurements of such systems typically require very long data acquisition times due to the low concentration and slow diffusion of the large particles. In order to overcome this drawback we apply hydrodynamic flow, which results in an increased flux of particles passing through the optical observation volume. This technique significantly reduces the data acquisition time of brightness experiments, while extending brightness analysis to femtomolar concentrations. The technique was developed using a test system of fluorescently labeled microspheres flowing through microfluidic channels to investigate the effect flow speed, particle size and brightness on FFS parameters. Finally this technique was extended to determine the copy number of fluorescently labeled Gag protein in viral-like particles present in cell medium. This work is supported by NIH grant R01GM064589.

3027-Pos

Characterization of Conjugated Protein by Molecular Brightness and Mass Spectrometry

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We have previously demonstrated characterization of antigen-antibody interactions with brightness analysis in fluctuation spectroscopy. In these experiments, the antigen studied was labeled with only a single fluorophore. In practice, it may not be straightforward to produce antigen with only a single fluorophore and random conjugation with a dye becomes the simplest approach. To use conjugated protein for single molecule studies, the distribution of conjugate must be understood. Here we introduce a method to evaluate conjugation of proteins using mass spectrometry. We define a mass spread function which describes the distribution of conjugate on a given protein. We show that convolution of this mass spread function with a protein's measured mass spectrum predicts the mass spectrum of conjugated protein. Application is shown using a highly glycosylated antibody with a low amount of incorporated conjugate. For measurement in solution, we use time integrated fluorescence cumulant analysis to characterize conjugated protein in terms of the molecular brightness. We then use brightness analysis to measure concentrations of free and bound conjugated protein in the presence of antibody.

3028-Pos

Combined Optical Tweezers and Fluorescence Microscopy for Single Molecule Experiments

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Optical traps (OT) and single molecule fluorescence (SMF) find use in single molecule biology [1,2]. OT allows manipulation and force spectroscopy of biological macromolecules. Unfortunately both the conformational state of the molecule and force localization on the molecular complex are unresolved in space. SMF characterizes the position of the fluorophore label, yielding the change in shape and conformational state of the labeled molecule. Correlating the force response and label tracking data, the relationship between changes in conformational state and related force are resolved [3]. A combined OT and SMF instrument (OT-SF) allow studying the operational principle of biological molecular motors essential to life.

We implemented SMF imaging into an existing optical tweezer instrument [4]. A diode pumped solid state laser excites fluorescence (Coherent Sapphire 50 mW, 488 nm). Video capture is realized with an intensified CCD camera (Qimaging QICAM Fast). A fluorescence emission filter (Chroma HQ535/30m) maximizes the SNR of fluorescence detection, by maximizing the optical density at the wavelengths corresponding to the trapping and detection lasers. A microscope TIRF objective (Nikon CFI Plan Apo 100X TIRF) facilitates localized excitation in the sample chamber, which reduces the background signal.

Proof-of-principle concurrent SMF imaging and OT micromanipulation of SYBR Gold stained DNA constructs is presented. The proof is presented in the form of a nanoscale video with fluorophore position and forces displayed in real-time. The DNA is bound to optically trapped dielectric beads in a dumbbell configuration.

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3029-Pos

Hyperspectral Line Scanning Microscopy for High-Speed Multicolor Quantum Dot Tracking

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One of the fundamental goals in observing protein-protein interactions on the cell membrane is in achieving nanometer scale spatial resolution along with temporal resolution sufficient to study live cell behavior. Traditional fluorescence microscopy methods have been unsuccessful in studying these interactions due to the diffraction limit with visible light. Single particle tracking techniques using quantum dots have provided single particle localizations to well below the diffraction limit, however, clustering of multiple particles limits the unique identification and thus tracking of individual particles throughout the (possibly dynamic) clustering process. This problem can be solved by tracking multiple quantum dot colors using a high-speed hyperspectral microscope which provides the necessary spatial, spectral, and temporal performance.

We describe a line scanning hyperspectral microscope that uses a prism spectrometer and a fast EMCCD camera to achieve 30 frames per second with 128 spectral channels. We present the optical setup, instrument control and display software and preliminary studies of multi-color quantum dot single particle tracking.

3030-Pos

A Time Resolved Fluorescence Spectrometer with Sub-Millisecond Data Acquisition Time

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We have developed a high-throughput time-resolved fluorescence spectrometer capable of recording a high-resolution time-domain (**sub-nanosecond**) fluorescence decay, with high S/N every 100 μs. Coupled with a conventional stopped-flow rapid mixer, this technology has allowed us to measure changes in time-resolved fluorescence decays occurring during the course of a millisecond-resolved biochemical transient experiment. Most instruments used in fluorescence-based kinetic studies are limited to detecting a single fluorescence intensity signal on the millisecond time scale. While this type of fluorescence intensity-based measurement is informative, it provides scant information compared to a full sub-nanosecond resolved fluorescence decay-which is exquisitely sensitive to the structure, dynamics, and interactions

of molecules in the sample. Current fluorescence lifetime technology is too slow by **a factor of at least 1000** to detect this decay accurately within the millisecond timescale of a typical biochemical transient. Our system, based on a direct acquisition data collection approach, records the entire fluorescence decay with S/N > 100 from a single pulse form a 10 kHz microlaser. Using this approach, we can resolve individual fluorescence lifetime components comprising as little as 10% of a complex multi-exponential fluorescence decay. When used to monitor structural transitions by time resolved FRET, we are capable of isolating individual structural states within complex structural ensembles. Using this approach we were able to simultaneously monitor the binding of myosin to different classes of binding sites on actin filaments. This type of analysis is not possible with conventional fluorescence based stopped flow measurements.

3031-Pos

Dual-Focus Confocal Microscopy for Flow and Brightness Measurements

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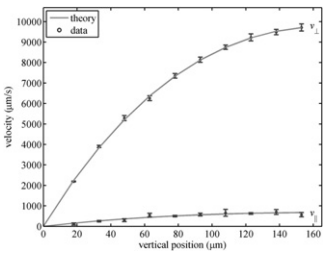
Fluorescence correlation spectroscopy (FCS) is a confocal microscopy method mostly used to measure the dynamic properties of molecules in solution and in biological membranes. In 2007, Dertinger *et al.* introduced a new two-beam confocal method called *dual-focus fluorescence correlation spectroscopy* (2f-FCS). [1] This development has been extremely important to the field because it overcomes major artifacts that limit conventional FCS as a quantitative measurement technique. In this poster we present two applications of 2f-FCS. One is measuring the velocity profile in a microfluidic channel with high accuracy and sub-micrometer spatial resolution. Second, we present a scheme to measure the absolute brightness of single fluorescent molecules, and thereby directly observe the heterogeneity intrinsic to biological systems. Measured velocity profile in a 300-by-300 micrometer-squared capillary.



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3032-Pos

Dual-Focus Fluorescence Correlation Spectroscopy: Measuring Translational and Rotational Diffusion of Biomolecules

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We present numerous applications of the recently developed dual-focus fluorescence correlation spectroscopy (2fFCS) to the measurement of translational diffusion of proteins and protein complexes. The method is applied to measuring Ca^{2+}-binding curves of wild-type and mutant of the proteins calmodulin and recoverin. 2fFCS is also capable of quantifying the conformational flexibility of macromolecular complexes, which is exemplified on the peptide binding of the major histocompatibility complex I (MHC I). Furthermore, we extended 2fFCS to measure fluorescence correlation at the nanosecond time scale, allowing also for measuring rotational diffusion. We performed a comparative study of translational and rotational diffusion (and related hydrodynamics size) of proteins, and present results for several globular proteins (BSA, human serum albumin, aldolase, ovalbumin). In all cases, measurements are performed at pico- to nanomolar sample concentrations, and with an accuracy of determining hydrodynamic size of a few percent. For performing 2fFCS measurements, a protein or protein complex needs only unspecific fluorescent labeling which is easily accomplished with commercially available dyes. Thus it is hoped that 2fFCS becomes a widely used and easy to handle technique in biophysical research.

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3033-Pos

Spatio-Temporal Control in Multiphoton Fluorescence Laser-Scanning Microscopy

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Confocal microscopy, one of the modern day fluorescence laser-scanning microscopic techniques, is suffered from having sufficient out-of-focus signal. On the other hand, in multiphoton microscopy ultrafast laser pulses are commonly used to circumvent low multiphoton absorption cross-sections of common fluorophores; due to broad overlapping two-photon absorption (TPA) spectra of fluorophores and large spectral bandwidth of a short pulse, simultaneous excitation of many fluorophores is common demanding selective excitation of individual fluorophores if required. Addressing the first issue, our recent work has shown that ultrafast one-photon pulsed illumination leads to increased signal-to-noise ratio by controlling the fluorophore photo-physics.[1-2] Considering TPA, we have demonstrated that photo-thermal corruption due to pulse pile-up effect is largely solvent-mediated and a rather slow process which can be taken care of by simple intensity modulation of a pulse-train.[3-4] We have recently shown how precise delay between pair of ultrafast pulses can lead to possible selective excitation in microscopy.[5] We have also demonstrated how gigantic peak-power of a femto-second laser pulse (with rather low average power) leads to stable optical trapping of latex nano-particles which is otherwise impossible with continuous-wave excitation (at the same average power).[6] All these cutting-edge topics will be discussed in the presentation.

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3034-Pos

Lifetime Resolved Fluorescence Fluctuation Spectroscopy

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Fluorescence correlation spectroscopy (FCS) has been widely used investigate molecular dynamics and interactions in biological systems. FCS typically resolves the component species of a sample either through differences in diffusion coefficient or molecular brightness. Diffusion based assays currently have a major limitation which requires that the diffusion coefficients of component species in a sample must be substantially different in order to be resolved. This criterion is not met in many important cases, such as when molecules of similar molecular weight bind to each other. This limitation can be overcome, and resolution of FCS measurements enhanced, by combining FCS measurements with measurements of fluorescence lifetimes. By using of global analysis on simultaneously acquired FCS and lifetime data we show that we can dramatically enhance resolution in FCS measurements, and accurately resolve the concentration and diffusion coefficients of multiple sample components even when their diffusion coefficients are identical provided there is a difference in the lifetime of the component species. We show examples of this technique using both simulations and experiments. It is expected that this method will be of significance for binding assays studying molecular interactions.

3035-Pos

Measuring Molecular Mobility with Fluorescence Anisotropy Macro Imaging

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Macro imaging systems with fields of view of tens of centimeters across are required for the whole body imaging of plants and small animals, but rarely have such systems been able to deliver molecular level information. We present a novel advancement to camera and lamp based macro imaging systems which introduces the capability of steady state anisotropy imaging. Our new fluorescence anisotropy macro imaging (FAMI) system provides the capability of rapidly and easily measuring molecular mobility both in-vitro and in-vivo. We show that the read-out on in-vitro assays can be highly quantitative. We also used FAMI to non-invasively examine the effects of temperature on live plants carrying fluorescent probes. The results indicate that low temperatures increase the internal viscosity of plants, and that the degree of increase is related to the anatomy of the plants. Our finding on plants verifies the results of others which showed that temperature and the water consumption of plant tissue are positively correlated and dependent on the tissue type. We also discuss further applications of FAMI to Förster Resonance Energy Transfer (FRET)-based sensors in assays and live animals.